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Hedgehog receptor function during craniofacial development

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ABSTRACT

The Hedgehog signalling pathway plays a fundamental role in orchestrating normal craniofacial development in vertebrates. In particular, Sonic hedgehog (Shh) is produced in three key domains during the early formation of the head; neuroectoderm of the ventral forebrain, facial ectoderm and the pharyngeal endoderm; with signal transduction evident in both ectodermal and mesenchymal tissue compartments. Shh signalling from the prechordal plate and ventral midline of the diencephalon is required for appropriate division of the eyefield and forebrain, with mutation in a number of pathway components associated with Holoprosencephaly, a clinically heterogeneous developmental defect characterized by a failure of the early forebrain vesicle to divide into distinct halves. In addition, signalling from the pharyngeal endoderm and facial ectoderm plays an essential role during development of the face, influencing cranial neural crest cells that migrate into the early facial processes. In recent years, the complexity of Shh signalling has been highlighted by the identification of multiple novel proteins that are involved in regulating both the release and reception of this protein. Here, we review the contributions of Shh signalling during early craniofacial development, focusing on Hedgehog receptor function and describing the consequences of disruption for inherited anomalies of this region in both mouse models and human populations.

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1. Introduction

The Hedgehog signalling pathway plays a key role in normal vertebrate development (Lee et al., 2016; Briscoe and Thérond, 2013; Ingham and McMahon, 2001) and maintenance of appropriate postnatal tissue homeostasis (Petrova and Joyner, 2014). In mammals, there are three Hedgehog-family members, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh); and amongst these, Shh has a predominant role in orchestrating developmental processes. The complexity of vertebrate Shh signalling has been highlighted by the identification of multiple novel proteins that are involved in mediating pathway transduction. Here, we review the contribution of Shh signalling during early craniofacial development, focusing on Hedgehog receptor function and the consequences of disruption for anomalies affecting this region in both vertebrate models and human populations.

2. Shh signal reception and transduction: an overview

Shh is released from the surface of signalling cells as a dual-lipidated protein modified by the addition of cholesterol and palmitate groups at the C and N-terminal regions, respectively (Pepinsky et al., 1998; Porter et al., 1995). Release from the environs of the cell is achieved through the combined activity of Dispatched (Disp), a multipass sterol-sensing domain protein (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002) and Scube2 (Signal peptide CUB EGF-like domain-containing protein), a secreted glycoprotein; both of which interact with Shh through its cholesterol moiety (Creanga et al., 2012; Tukachinsky et al., 2012) (Fig. 1). Once released, Shh can signal within embryonic tissues at both short and long-range (Gritli-Linde et al., 2001).

In recent years, the primary cilium has been identified as a key multifunctional organelle involved in the essential regulation of numerous signalling pathways, including vertebrate Hedgehog (Berbari et al., 2009; Huangfu and Anderson, 2005; Pedersen and Rosenbaum, 2008) (Fig. 1). Indeed, ciliopathies are now recognized as a significant group of disorders that arise from defective function of these organelles (Badano et al., 2006), which often involve

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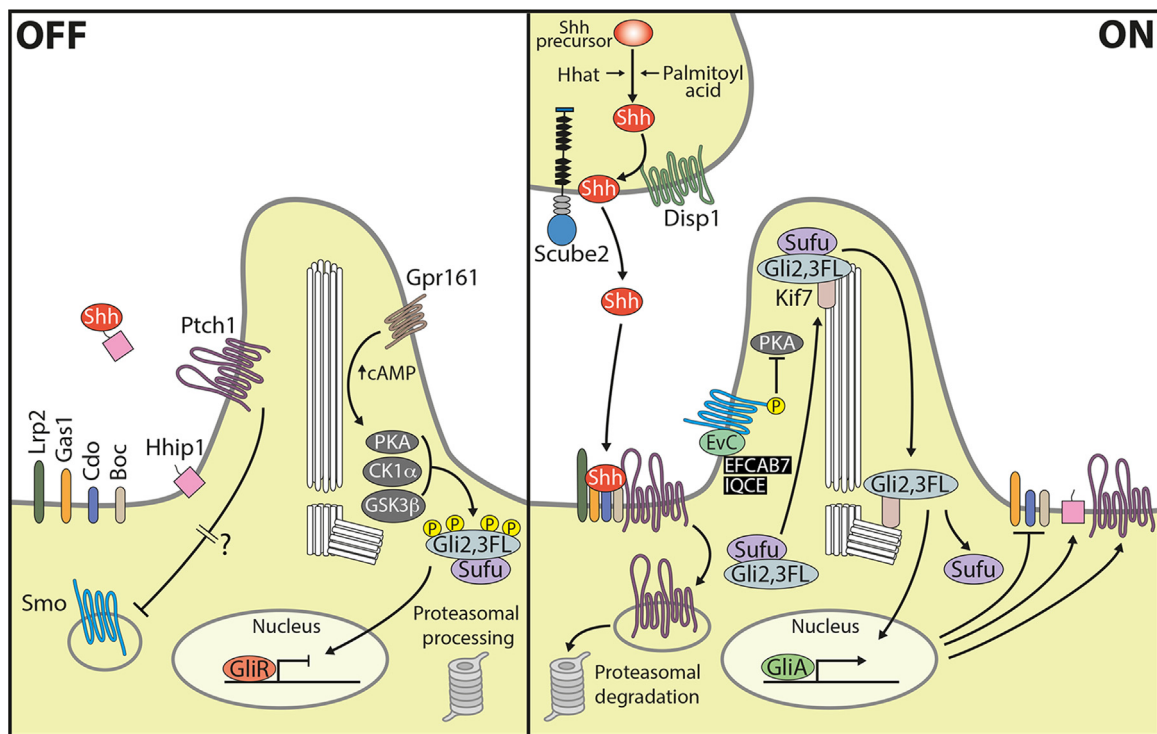


Fig. 1. Shh signalling pathway. In the absence of Shh (OFF, left panel) Ptch1 predominates in the primary cilium membrane and represses Smo through an uncharacterized (but indirect) mechanism that prevents Smo enrichment at the cilium. Gpr161 is also localized to the cilium and may contribute to activation of PKA by generating an increase in cAMP. Full length Gli2/3 proteins are maintained in the cytosol by SuFu, which exposes them to multiple phosphorylation by PKA, CK1α and GSK3β and their partial degradation (through proteolytic processing) into truncated GliR repressors that suppress Shh target gene transcription in the nucleus. In the presence of Shh (ON, right panel) ligand binding to Ptch1 takes place, which is facilitated by formation of a Lrp2/Boc/Cdon/Gas1 multi-molecular co-receptor complex. Ptch1 exits the cilium and relief of Smo inhibition occurs. Smo is phosphorylated by CK1α and the GPCR kinase-2 (GRK2) (not shown) and enriches in a signalling microdomain at the base of the cilium through formation of an EvC complex involving the ciliary proteins EFCAB7 and IQCE. This results in Smo inhibition of PKA and facilitates Kif7-mediated anterograde transport of a full length Gli2/3-SuFu complex to the tip of the cilium, where it dissociates from the negative regulator SuFu. Gpr161 is removed and internalized from the cilium, which prevents its inhibitory activity (not shown). The production of Gli2/3 activators (GliA) derived from the full-length Gli proteins occurs, and accumulation of GliA in the nucleus enables activation of Shh target genes, such as *Ptch1*, *Gli1* and *Hhip1*, and the inhibition of *Gas1*, *Cdo* and *Boc*. In secreting cells, Shh undergoes a series of internal modifications before reaching an active, multimeric form. Hhat is necessary for the post-translational palmitoylation of Shh; in the absence of Hhat, Shh secretion is decreased. Shh first traverses the lipid bilayer via its interaction with Disp1 and then is extracted from the membrane via interaction with Scube2. Exactly how Scube2 facilitates the subsequent movement of Shh away from its source remains unclear. Boc, Boc cell adhesion associated oncogene regulated protein; cAMP, Cyclic adenosine monophosphate; Cdon, Cell adhesion associated, oncogene regulated protein; CK1α, Casein Kinase 1 alpha; Disp1, Dispatched 1; EFCAB7, EF-hand calcium-binding domain-containing protein7; EvC, Ellis-van Creveld transmembrane protein complex (EvC and EvC2); Gas1, Growth-arrest specific gene 1; Gli, Glioma-associated oncogene family member (A, active form; R, repressor form; FL, full length); Gpr161, orphan rhodopsin GPCR 161; GSK3β, Glycogen synthase kinase 3 beta; Hhat, Hedgehog acyltransferase; Hhip1, Hedgehog-interacting protein 1; IQCE, IQ-domain containing protein E; Kif7, Kinesin family 7; P, phosphorylation; PKA, Protein Kinase A; Ptch1, Patched 1; Lrp2, low-density lipoprotein receptor-related protein; Scube2, Signal peptide CUB EGF-like domain-containing protein 2; Shh, Sonic hedgehog; Smo, Smoothened; SuFu, Suppressor-of-fused.

disrupted Hedgehog signalling and have an affect on the craniofacial region (Brugmann et al., 2010; Cortes et al., 2015). Reception at target cells is mediated primarily through Patched1 (Ptch1), a twelve-pass transmembrane protein, which functions as a ligand-independent inhibitor in the resting state (Goodrich et al., 1996). Additional receptor proteins have also been identified that can physically associate with Shh at the cell surface, which include the low-density lipoprotein receptor-related protein, Lrp2 (otherwise known as gp330/megalin) (Saito et al., 1994), the GPI-linked membrane glycoprotein Growth arrest-specific1 (Gas1) (Martinelli and Fan, 2007) and the Ig/fibronectin single-pass membrane-spanning cell adhesion proteins Cdon (cell adhesion associated, oncogene regulated) and Boc (Boc cell adhesion associated, oncogene regulated) (Kang et al., 2002, 1997).

In the absence of Shh, Ptch1 accumulates in and around the cilium and represses the activity of Smoothened (Smo), a G-protein coupled receptor (GPCR)-like protein (Corbit et al., 2005; Rohatgi et al., 2007) absolutely required for signal transduction (Zhang et al., 2001). The precise mechanism of Smo function in transduction is poorly understood, with no evidence that it represents a true GPCR (Wang et al., 2013) or indeed, that GPCR signalling events contribute to signal transduction (Low et al., 2008). However, the orphan rhodopsin GPCR Gpr161 does localize

to the cilium and acts to negatively regulate the pathway through the activation of Protein Kinase A (PKA) (Hwang and Mukhopadhyay, 2014; Mukhopadhyay and Rohatgi, 2014; Mukhopadhyay et al., 2013). Ptch1-mediated inhibition of Smo is only relieved by the direct binding of Shh (Stone et al., 2006); which results in the internalization and degradation of Shh-Ptch1 (Incardona et al., 2000) and removal of Gpr161 (Mukhopadhyay et al., 2013). Smo becomes phosphorylated in a Hedgehog-dependent manner by Casein Kinase 1α (CK1α) and the GPCR Kinase (GRK2) (Chen et al., 2004; Evangelista et al., 2008; Meloni et al., 2006) and accumulates at the ciliary membrane (Chen et al., 2011; Corbit et al., 2005; Rohatgi et al., 2009). Current models suggest that Ptch1 regulates Smo indirectly (Taipale et al., 2002), with Smo enrichment in the ciliary membrane correlating with signal activation (Barakat et al., 2013; Firestone et al., 2012; Nedelcu et al., 2013; Ocbina and Anderson, 2008; Ocbina et al., 2011; Rohatgi et al., 2009). However, Smo constantly cycles through the cilium and accumulation can also occur in the absence of signal when retrograde ciliary trafficking through the dynein2 microtubule motor is impaired (Kim et al., 2009). Indeed, it has been demonstrated that whilst ciliary accumulation of Ptch1 is absolutely required for Smo suppression, Smo removal is not; seemingly fine-tuning, rather than being essential for pathway activity (Kim et al., 2015).

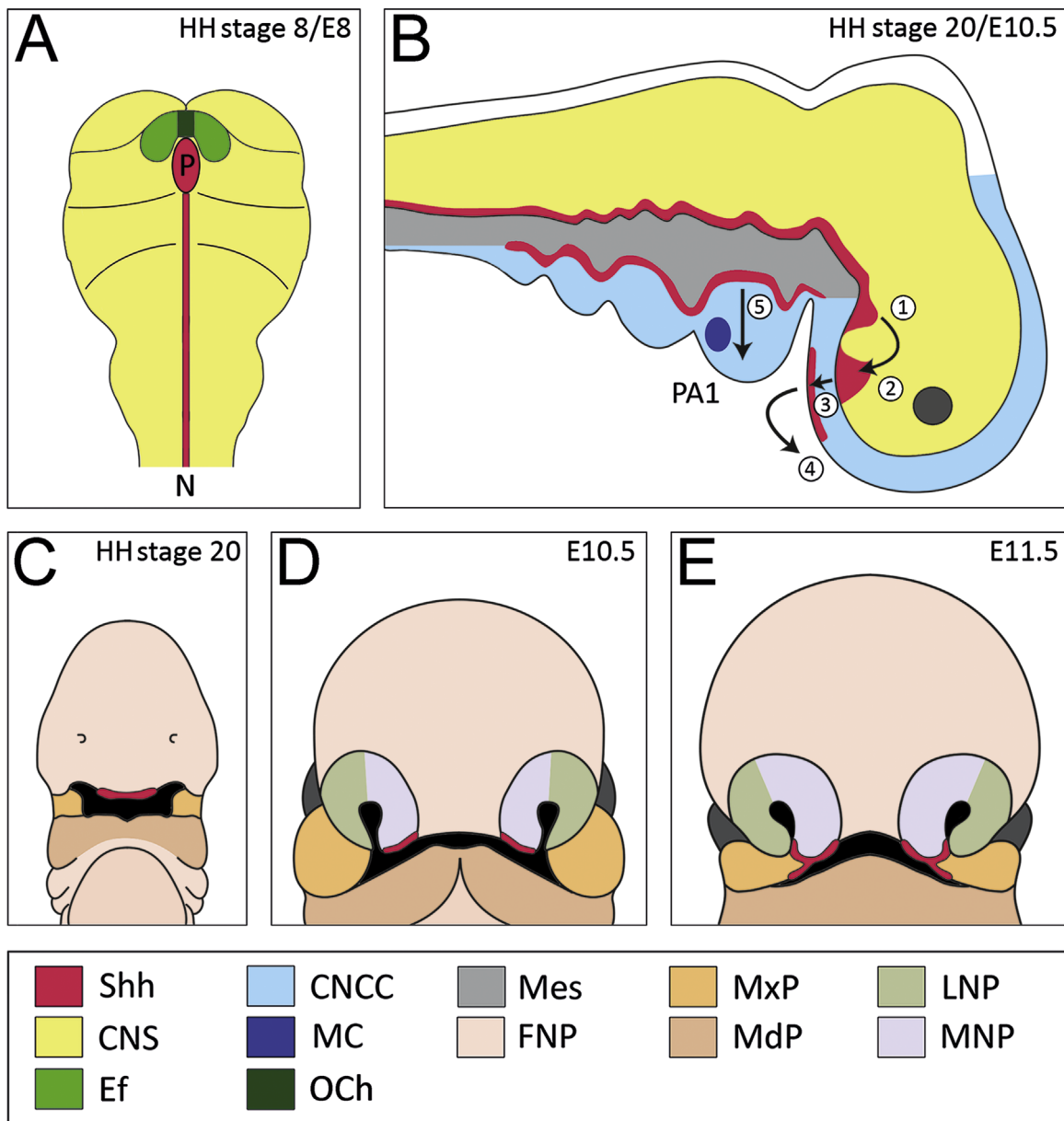


Fig. 2. Shh signalling during early craniofacial development. (A) An early essential role for Shh is through signalling from the prechordal plate, which underlies the prosencephalic neural plate and is required for normal division of the eye field (Hamburger Hamilton (HH) stage 6–8 in the chick and E8 in the mouse). (B) At later stages, Shh is present in three ectodermal domains in the early head: neuroectoderm of the ventral forebrain, ectoderm of the facial midline and pharyngeal endoderm (HH stage 20 in the chick and E10.5 in the mouse). Reiterative Shh signalling within the early forebrain from the diencephalon (1) to telencephalon (2) establishes *Shh* in the facial ectoderm (3). This domain plays a key role in establishing appropriate polarity and growth of the facial processes (4). Shh signalling from the pharyngeal endoderm is also essential for growth and survival of cranial neural crest cells populating the first pharyngeal arch and the formation of Meckel's cartilage (5). (C–E) Shh signalling domains in the developing face. (C) Shh is present in midline ectoderm of the frontonasal process in the early chick embryo (HH stage 20); (D) whilst in the mouse, signalling is restricted to the medial nasal processes bilaterally from around E10 and firmly established at E10.5 (shown here); (E) extending into the base of the nasal pit and maxillary process during formation of the upper lip at E11.5. Levels of Shh signalling in the medial nasal process is crucial for normal lip fusion, being required to inhibit canonical WNT signalling in the lateral nasal process and epithelial seam. Shh, sonic hedgehog expression; CNS, central nervous system; Ef, eye field; CNCC, cranial neural crest cells; MC, Meckel's cartilage; OCh, optic chiasma; Mes, mesoderm; FNP, frontonasal process; MxP, maxillary process; MdP, mandibular process; LNP, lateral nasal process; MNP, medial nasal process; PA1, first pharyngeal arch; P, prechordal plate; N, notochord. (A) is adapted from Rubenstein and Beachy (1998) and (C, D) from Hu and Marcucio (2009a).

Downstream intracellular signalling is subsequently coordinated directly within the cilium through processing of Gli (Glioma-associated oncogene family members; Gli1–3) transcription factors and their entry into the nucleus (Haycraft et al., 2005). Gli proteins provide a read-out for transcriptional activation or repression, primarily through the relative levels of Gli2 activator and Gli3 repressor function (Aberger and Ruiz, 2014; Hui and Angers, 2011). Gli activity is ultimately controlled by multiple degradation mechanisms: in the absence of signal, Gli3 (and Gli2) undergo partial proteolysis into truncated repressor forms [GliR],

with Gli3R acting as the principal repressor; whilst in the presence of signal, full length Gli2 (and Gli3) are preserved in their active forms [GliA], with Gli2 acting as the principal activator. Gli1 is a direct transcriptional target of signalling and amplifies transcriptional output, but is not essential for development (Bai et al., 2002; Park et al., 2000).

Within the cilium, activated Smo associates with the type I single-pass transmembrane domain proteins EvC and EvC2 (the causative genes in Ellis-van Creveld syndrome) to form a complex tethered within a basal EvC zone. This complex is required for

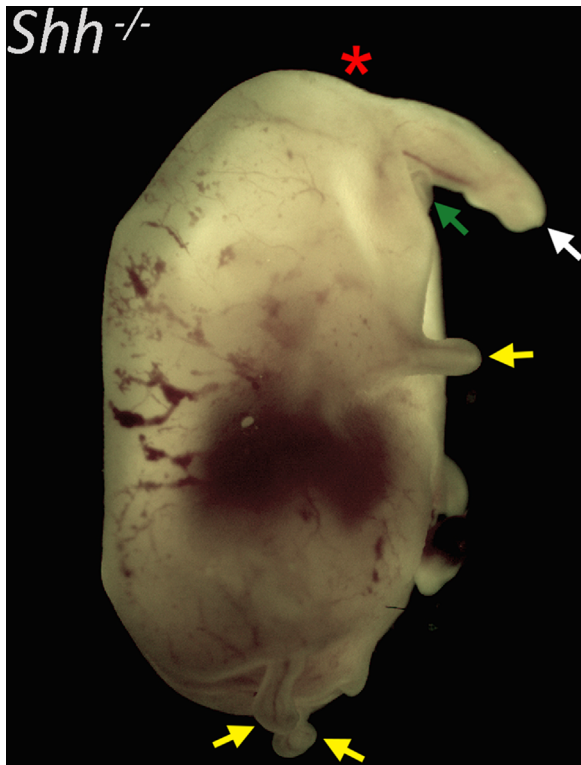


Fig. 3. Loss of Shh function produces holoprosencephaly. E14.5 *Shh*^{-/-} mouse embryo. There is alobar holoprosencephaly, with the forebrain remaining as a single vesicle (red *) and the craniofacial region dominated by the presence of a large midline proboscis (white arrow) situated above a single midline ocular region (green arrow). The pharyngeal arches are absent and limb development is severely disrupted (yellow arrows).

surface, and pathway activity is buffered in a ligand-dependent manner (Casali and Struhl, 2004; Chen and Struhl, 1996). The relative availability of bound and unbound receptor is further regulated through transcriptional repression of the Hedgehog coreceptors *Gas1*, *Cdon* and *Boc* (Martinelli and Fan, 2007; Tenzen et al., 2006).

3. Shh signalling: a key player in craniofacial development

Shh is present throughout the axial mesendoderm of the early embryo, including the prechordal plate; and plays an essential role in establishing subdivision of the eyefield and bilateral patterning of the ventral forebrain (Fig. 2A) (Chiang et al., 1996). At later stages, Shh signals from three key ectodermal domains in the early head, neuroectoderm of the ventral forebrain, ectoderm of the facial midline and pharyngeal endoderm, with signal transduction evident in both the ectodermal and underlying mesenchymal tissue compartments, including cranial neural crest cells (CNCC) (Fig. 2B). Signalling is then seen within the facial processes during early formation of the upper lip and palate. Collectively, these domains of Shh signalling activity have an intimate temporo-spatial relationship and their coordinated function is essential for orchestrating fundamental organization of the craniofacial region, particularly structures within the midline (Fig. 2C–E) (Helms et al., 2008; Marcucio et al., 2011; Petryk et al., 2015; Tapadia et al., 2005). Mice generated with targeted deletion of *Shh* have significant craniofacial defects, including alobar holoprosencephaly, cyclopia (Chiang et al., 1996), failure of the primary mouth (stomodeum) to open (Tabler et al., 2014) and hypoplasia of the first pharyngeal arch (Washington Smoak et al., 2005; Yamagishi et al., 2006).

3.1. Holoprosencephaly

An early discovery was the key role that Shh plays during development of the forebrain; in particular, the association between signalling and the etiology of holoprosencephaly (HPE) (Fig. 3) (Chiang et al., 1996). HPE is a clinically heterogeneous developmental defect characterized by a failure of the early forebrain vesicle to divide into distinct halves (Geng and Oliver, 2009). The underlying brain malformation can have a significant influence upon subsequent development within the facial midline. In the alobar form, the forebrain remains as a single undivided vesicle accompanied by cyclopia, where a single midline eye is situated below a proboscis. In semilobar HPE there is a partial separation of the forebrain hemispheres; whilst in the lobar form, hemispheric separation is essentially normal. In microforms of HPE, the central nervous system (CNS) is normal with only milder craniofacial features, such as ocular hypotelorism, pre-maxillary agenesis and solitary median maxillary central incisor (SMMCI) (Ming and Muenke, 2002). Shh signalling from the prechordal plate and ventral midline of the diencephalon is required for appropriate division of the forebrain vesicle and in humans, mutation in a number of SHH pathway components have been associated with various forms of HPE. These include *SHH* itself (Belloni et al., 1996; Roessler et al., 1996), *DISP1* (Belloni et al., 1996; Roessler et al., 1996, 2009), *PTCH1* (Ming et al., 2002), *GAS1* (Ribeiro et al., 2010), *CDON* (Bae et al., 2011) and *GLI2* (Roessler et al., 2003). However, the etiology of HPE is complex, with both genetic and environmental factors being implicated (Ming et al., 1998). Moreover, a range of severity can exist in both the brain malformation and craniofacial features that are seen, with clinical variability a characteristic feature of this condition (Petryk et al., 2015).

signal transduction in a tissue-specific manner and acts upstream of the negative Hedgehog regulators PKA and the tumour-suppressor protein Suppressor-of-fused (Sufu) (Blair et al., 2011; Caparros-Martin et al., 2013; Dorn et al., 2012; Yang et al., 2012). Sufu is a major negative regulator of Hedgehog, acting in the cilium to bind and sequester Gli proteins and control their levels, particularly Gli2 and Gli3A (Ding et al., 1999; Humke et al., 2010; Koger et al., 1999; Pearce et al., 1999; Stone et al., 1999; Tukachinsky et al., 2010); but also regulating Gli transcriptional activity directly in the nucleus (Lin et al., 2014). PKA also negatively regulates signalling at the base of the cilium, achieving distinct patterns of phosphorylation on full length Gli2/3 to initiate a pathway of proteasomal processing into truncated GliR forms (Niewiadomski et al., 2014; Pan et al., 2009; Tuson et al., 2011; Wang et al., 2000; Wen et al., 2010). Activated Smo promotes the transport of Gli-Sufu to the tip of the cilium and inhibits activity of both Sufu and PKA, leading to dissociation of Gli from Sufu, inhibition of GliR function and entry of GliA into the nucleus (Kim et al., 2009; Niewiadomski et al., 2014; Wen et al., 2010). In addition, the kinesin family Kif7 ciliary protein is a further regulator of signalling, involved in facilitating Gli processing at the tip of the cilium. In the presence of Hedgehog, Kif7 enriches at the tip of the cilium and acts on microtubules to maintain appropriate architecture of these structures (He et al., 2014). In the absence of Kif7 function, Gli3R levels are reduced and Gli2A activity is increased, which results in mildly elevated signalling (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009).

Amongst the direct transcriptional targets of Shh are *Gli1*, *Ptch1* and Hedgehog-interacting protein (*Hhip1*), a membrane glycoprotein that can also bind and sequester Shh (Chuang and McMahon, 1999). Thus, a caveat of signal activation is that *Ptch1*, *Patched 2* (*Ptch2*) (and *Hhip1*) rapidly accumulate on the cell

3.2. Cranial neural crest cells

There is good evidence that Hedgehog transduction is important for the survival of CNCC, with function-blocking experiments in the chick resulting in premature death of this cell population (Ahlgren and Bronner-Fraser, 1999). Survival in the first pharyngeal arch is promoted by Shh acting from ventral foregut endoderm (see below) at least partially through the inhibition of Cdon, which acts as a dependence receptor to promote apoptotic activity in this cell population (Delloye-Bourgeois et al., 2013). In the zebrafish, Hedgehog signalling from the ventral brain primordium has an early indirect role in orchestrating craniofacial development, establishing the stomodeum, which subsequently interacts in a Hedgehog-independent manner with CNCC to generate the pterygoid process of the quadrate in the upper jaw and the anterior neurocranium (Eberhart et al., 2006). Hedgehog signalling also becomes established in oral ectoderm of the zebrafish, acting directly to specify the movement of distinct CNCC populations forming the main cartilaginous elements of the anterior neurocranium and regulating their differentiation into cartilage (Wada et al., 2005). In addition, Hedgehog signalling to CNCC in the pharyngeal arches is further required to maintain *shh* expression in the pharyngeal endoderm of zebrafish (Swartz et al., 2012)

Loss of Hedgehog signalling in murine CNCC through *Wnt1Cre*-mediated targeted disruption of *Smo* (*Wnt1Cre; Smo*) function has also demonstrated the importance of normal Shh responsiveness in these cells during craniofacial development. A number of CNCC-derived skeletal and non-skeletal components are absent in these mice, which have facial truncation due to reduced growth and development of the pharyngeal arches secondary to increased apoptosis and decreased cell proliferation (Jeong et al., 2004). Moreover, the catastrophic loss of architecture seen in pharyngeal arch I of *Shh* mutant embryos may also compromise the domains of CNCC populations within this region (Washington Smoak et al., 2005; Yamagishi et al., 2006). Interestingly, CNCC migration appears to be normal in the absence of Hedgehog-responsiveness in CNCC (Jeong et al., 2004), consistent with findings in zebrafish *smo* and *disp1* mutants (Eberhart et al., 2006; Schwend and Ahlgren, 2009); however, zebrafish *cdon* does play a role in mediating the migration of trunk NCC through the regulation of N-cadherin localization (Powell et al., 2015).

More recently, it has been shown that mice with loss-of-function mutation in the Fuzzy ciliogenesis regulator have high-arched palate, which is a common feature amongst human ciliopathies. Interestingly, whilst there is dysregulated Gli processing and reduced Hedgehog signalling in these mice, the phenotype arises through upregulated *Fgf8* expression early in development and

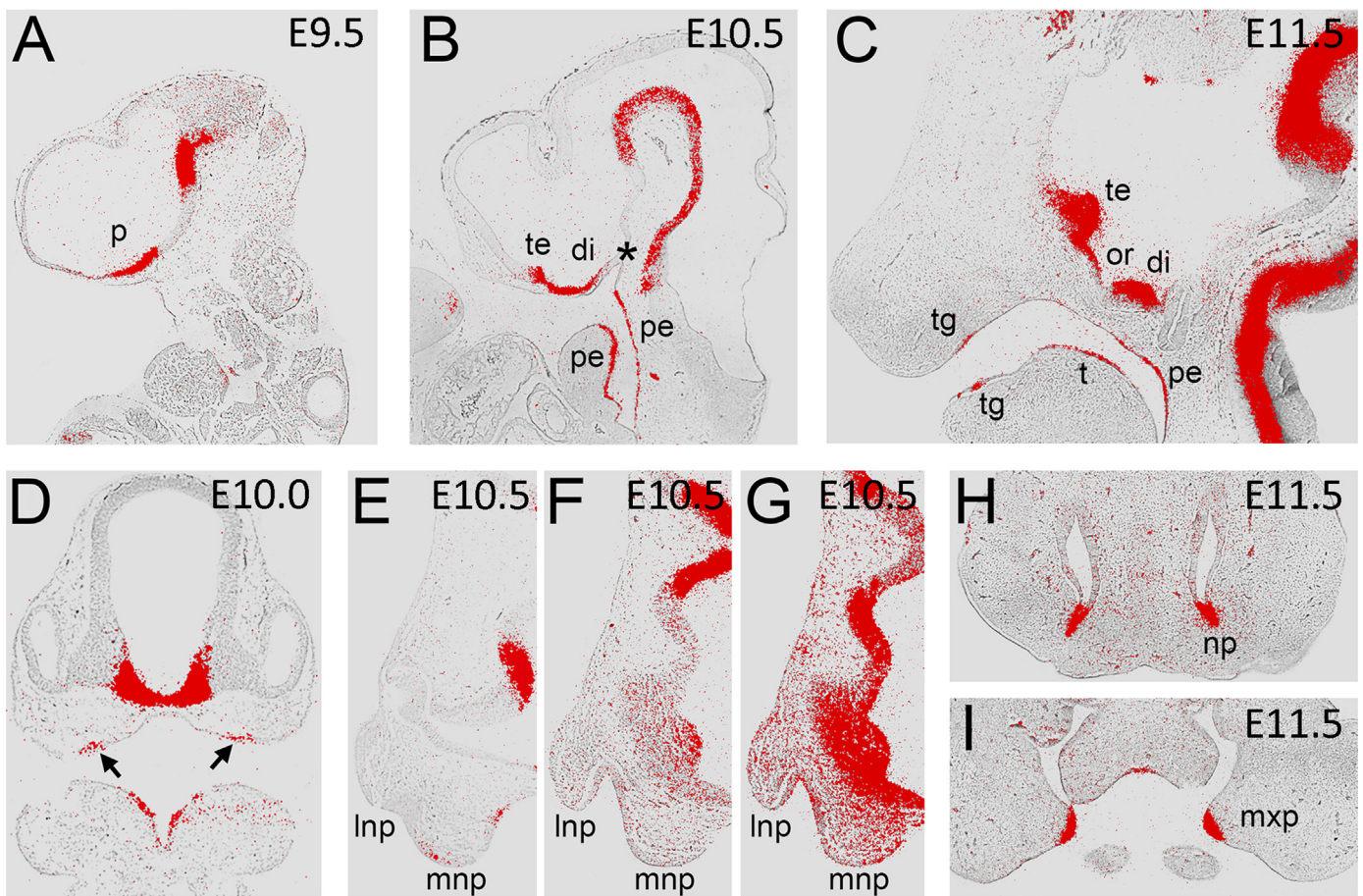


Fig. 4. Shh pathway expression domains in the murine craniofacial region. (A–C) Sagittal sections through the developing craniofacial midline. (A) At E9.5, *Shh* is expressed in the ventral prosencephalon; (B) By E10.5, transcripts are localized to the ventral diencephalon, telencephalon and pharyngeal endoderm, but are absent from ectoderm of Rathke's pouch (*); (C) At E11.5, the domains within the diencephalon and telencephalon are well-established and separated by the optic recess, with expression also present in ectoderm of the early tongue and the developing incisor tooth germs. (D–I) Frontal sections through the early facial region. (D) At E10.0, *Shh* is expressed bilaterally in ectoderm of the early medial nasal processes (arrowed); (E, F) At E10.5, *Shh* is expressed in ectoderm of the medial nasal processes in deeper regions of the face but not more superficially; however, a gradient of signalling activity exists in the medial nasal process at this stage, running from medial to lateral, as demonstrated by *Ptch1* expression in this region (G). (H, I) At E11.5, *Shh* is expressed in ectoderm at the base of the nasal pits and in the maxillary processes during formation of the upper lip. di, diencephalon; or, optic recess; p, prosencephalon; pe, pharyngeal endoderm; t, tongue; te, telencephalon; tg, tooth germ; Inp, lateral nasal process; mnp, medial nasal process; mxp, maxillary process; np, nasal pit.

increased numbers of CNCC in the maxillary process. A similar phenotype is seen in oral-facial-digital syndrome (*Odf1*) mutant mice, which also have defective ciliogenesis, suggesting shared developmental mechanisms between the ciliopathies and Fgf-related craniofacial syndromes (Tabler et al., 2013).

3.3. Facial development

Shh expression in surface ectoderm of the facial processes is dynamic and reflects the capacity of this pathway to organize and directly influence formation of this region (Helms et al., 1997; Hu and Helms, 1999) (Fig. 4).

In the chick (see Fig. 2C), *Shh* is initially expressed in midline ectoderm of the frontonasal process (FNP), forming a boundary with *Fgf8*-expressing cells situated more dorsally, in a so-called frontonasal ectodermal zone (or FEZ), which acts as a signalling centre to control growth and polarity within the upper jaw (Abzhanov and Tabin, 2004; Hu and Marcucio, 2009a, 2009b; Hu et al., 2003). A FEZ is also present in mice (see Fig. 2D), but the domains of *Shh* are restricted to bilateral regions of ectoderm within the early medial nasal processes (MNP), later extending into the base of the MNP and nasal pit, and appearing in the maxillary processes as the upper lip is formed (see Fig. 2E). These domains subtly direct appropriate growth and cell survival within these regions during the establishment of early facial form (Hu et al., 2015). Interestingly, all the primary *Shh* signalling domains within the early craniofacial midline are reciprocal and closely coordinated, particularly those present at very early stages between the developing forebrain and facial region (Marcucio et al., 2011) (Fig. 4A–C). Early expression of *Shh* is seen in the ventral prosencephalon and diencephalon, subsequently inducing a domain in the basal telencephalon, anterior to the optic recess; and in the chick, establishing competency of the FEZ to express *Shh* (Hu and Marcucio, 2009a, 2009b; Marcucio et al., 2005). *Shh* signalling between the brain and face therefore follows a very specific temporo-spatial pattern, influencing outgrowth and patterning of the upper midface (Chong et al., 2012). Indeed, this signalling activity in the embryonic midface has been shown to have a predictive relationship for shape variation within the upper jaw of the chick. This dose-response is non-linear and consistent with *Shh* acting through a morphogenetic gradient (Young et al., 2010).

Collectively, these findings suggest a basis for the wide range of severity that is seen in conditions such as HPE, particularly in relation to the midline facial anomalies. Variation in the temporal activity of this pathway providing some context to the phenotypic spectrum that is often observed (Cordero et al., 2004).

3.4. Oro-facial clefting

Shh is expressed in regionally-restricted domains of the early facial processes (Fig. 4D–I) and later in the palatal shelves, which is consistent with a role in development of the upper lip (Kurosaka, 2015) and secondary palate (Cobourne and Green, 2012). Pharmacological antagonism of Hedgehog signalling in the mouse embryo between E7–9 produces cleft lip and palate (CLP), whilst between E9–10 it causes isolated cleft palate (CP) (Heyne et al., 2015; Lipinski et al., 2010).

There is evidence that *Shh* interacts with Bone morphogenetic protein (Bmp), Fibroblast growth factor (Fgf) and Wntless-type MMTV integration site (Wnt) pathways in a dynamic and rapidly-changing manner during development of the murine upper lip (Kurosaka et al., 2014; Metzis et al., 2013; Thomason et al., 2008). Around E10.5–11, a gradient of Hedgehog signal activity extends from the facial midline across the MNP's (Fig. 4E–G), with loss of *Ptch1* in CNCC resulting in increased transduction and reduced Fgf signalling in the facial prominences, associated with defective

nasal pit invagination and cleft lip (Metzis et al., 2013), and consistent with associations between *PTCH1* mutations and CLP in human populations (Letra et al., 2010; Mansilla et al., 2006; Sasaki et al., 2009). At E11, *Shh* signal activity within the MNP represses canonical Wnt signal transduction in regions cranial to the lambdoid region and epithelial seam. This facilitates Wnt-mediated induction of *p63* and *Irff6* in this region, and appropriate removal of the epithelial seam during upper lip formation (Kurosaka et al., 2014). Interestingly, at E11.5 *Shh* becomes strongly expressed in ectoderm of the caudal MNP and adjacent maxillary process – regions of expression that are downregulated and lost, respectively in the *p63* mutant (Thomason et al., 2008).

Shh is also expressed on the oral surface of the murine secondary palate from around E12 in the mouse, in a series of eight stripes that correspond to the future *rugae palatinae* (Pantalacci et al., 2008; Welsh and O'Brien, 2009). *Shh* signalling is required from epithelium to mesenchyme for appropriate proliferation in the mesenchymal compartment of the palatal shelves and disruption produces CP (Dassule et al., 2000; Jeong et al., 2004; Lan and Jiang, 2009), although with ablation of *Shh*-responsiveness in the epithelium alone, the palate remains intact (Gritli-Linde et al., 2002). *Shh* lies downstream of Wnt signalling in the oral epithelium (Lin et al., 2011) and participates in reciprocal induction of *Fgf10* in the mesenchyme, mediated through *Fgfr2b* function in the epithelium, with loss of both these Fgf signalling components also associated with CP (Rice et al., 2004). Further associations between perturbed *Shh* signalling and oro-facial clefting are seen in *Cdon* and *Gas1* mutant mice, which exhibit philtral dysgenesis and CP, respectively in a 129sv/C57BL/6 background (Cole and Krauss, 2003; Seppala et al., 2007); whilst *Gas1*; *Boc* mutant mice have CLP associated with increased cell death and reduced proliferation in the facial midline (Seppala et al., 2014).

The eight *rugae palatinae* appear sequentially from within a growth zone situated in the mid-palate, anterior to the first-forming ruga 8. Subsequent growth of the palatal shelves and appearance of the rugae is closely co-ordinated, with ruga 2 appearing next, followed by rugae 3, 1 and then 4–7 (Economou et al., 2012; Pantalacci et al., 2008; Welsh and O'Brien, 2009). It has been suggested that the regular spacing between rugae is established through an activator-inhibitor mechanism, with Fgf and Wnt signalling acting as the activator component and *Shh* as an inhibitor (Economou et al., 2012).

3.5. Pharyngeal endoderm

Shh is also strongly expressed within the pharyngeal endoderm (see Fig. 4B, C), directly regulating normal morphogenetic movement of the pharyngeal arches in zebrafish (Swartz et al., 2012). In the chick, it is required for cell survival and proliferation within the mandibular primordium (Brito et al., 2006; Couly et al., 2002; Haworth et al., 2007a) and induction of the mesethmoid cartilage in the most rostral-zone (Benouaiche et al., 2008). In the mouse, conditional inactivation of *Shh* in the pharyngeal endoderm leads to micrognathia secondary to increased mesenchymal cell death in pharyngeal arch I (Billmyre and Klingensmith, 2015). These mice also have significant pattern defects within the first arch, consistent with observations that *Fgf8* is downstream of *Shh* in this region (Haworth et al., 2007a). A failure of Meckel's cartilage differentiation also occurs in the absence of *Shh* signalling from the endoderm (Billmyre and Klingensmith, 2015), whilst a source of ectopic *Shh* in mandibular arch ectoderm or mesenchyme can induce an ectopic or supernumerary Meckel's cartilage in the chick, which can produce mirror-image supernumerary jaws (Brito et al., 2008; Haworth et al., 2007a). Interestingly, *Gas1*^{−/−}; *Shh*^{+/-} compound mutant mice have ectopic duplications of the proximal mandible that include the molar dentition, a finding

seemingly difficult to reconcile with reduced Hedgehog signalling in this region (Seppala et al., 2007). In addition, transgenic mice overexpressing *Shh* in the oral epithelium through a *Keratin14* promoter have essentially normal morphology of the mandible, apart from some diminution of the coronoid process (Cobourne et al., 2009).

4. Hedgehog receptor function in craniofacial development

In recent years, progress has been made in further understanding the role of multiple proteins that interact with Shh during normal signal transduction. In particular, these proteins are intimately involved in both the production and reception of Shh during development. Here, we focus on the function of these diverse proteins within the context of Shh signalling during craniofacial development and the consequences of disrupted function for normal development of this region (Table 1).

5. Hedgehog production

5.1. Dispatched

There are two *Disp* homologues in mouse (*Disp1* and *Disp2*), but genetic studies support the view that only *Disp1* is involved in signalling, with mutants failing to survive beyond embryonic day (E) 9.5 and presenting with abnormal morphology of the forebrain (indicative of a loss of ventral midline fate) and delayed cardiac morphogenesis (Casparly et al., 2002; Kawakami et al., 2002; Ma et al., 2002). *Disp1* activity is required in Shh-producing cells for paracrine signalling through cholesterol-modified Shh (Tian et al., 2005). Indeed, during facial development attenuating *Disp1* activity using both hypomorphic and missense alleles produces a loss of midline facial structures, with a dose-dependent genetic interaction existing between *Shh* and *Disp1*. The severity of the facial anomalies is reflected in the amount of disruption seen within the premaxilla, and both truncation and fusion in the mandibular region, with the most severe embryos lacking a premaxilla and the mandibular incisor dentition (Tian et al., 2005). Inactivation of zebrafish *disp1* using the *chameleon* (*con/displ*) mutant results in a significant, but incomplete reduction in Hedgehog signalling. These mutants demonstrate a requirement for *disp1* in patterning post-migratory CNCC, specifically in pharyngeal arch I and regulating the chondrogenic markers *dlx2a* and *sox9a*. In addition, patterning of the jaw joints is severely disrupted, through loss of *bapx1* and *gdf5* (Schwend and Ahlgren, 2009).

Significantly, truncating mutations of *DISP1* have been identified in two independent human families, where affected individuals demonstrated clinical features of microform HPE (Roessler et al., 2009). *DISP1* interacts with human SHH via its cholesterol anchor, and this interaction is necessary for appropriate SHH secretion. However, *DISP1* alone is not sufficient to release SHH from cells, this process also requires SCUBE2 function in order to overcome the insolubility conferred by SHH cholesterol modification (Creanga et al., 2012; Tukachinsky et al., 2012).

5.2. Scube2

The Scube gene family consists of three independent evolutionarily conserved members (Scube1–3) (Haworth et al., 2007b; Hollway et al., 2006; Woods and Talbot, 2005; Wu et al., 2004, 2011; Xavier et al., 2013, 2009; Yang et al., 2002). These genes encode secreted and cell surface-associated proteins that share a domain organization of at least five recognizable motifs, including

multiple EGF (epidermal growth factor-like) and N-linked glycosylation sites, and a C-terminal CUB domain (Grimmond et al., 2000). Zebrafish *scube2* plays a non-cell-autonomous role in mediating long-range Hedgehog signalling, with mutants having morphological traits consistent with reduced pathway activity, such as a curled tail, U-shaped somite boundaries and significantly, weak cyclopia (Hollway et al., 2006; Johnson et al., 2012; Kawakami et al., 2005; Woods and Talbot, 2005). A more severe loss of Hedgehog target gene expression is observed when all *Scube* family members are knocked down; however, defects in Hedgehog-sensitive cell types are also evident when *scube1* or *scube3* are lost in combination with *scube2*. These findings suggest that *scube2* is the most important family member in modulating Hedgehog signalling (Johnson et al., 2012), an observation consistent with the grossly normal craniofacial development of murine *Scube3* mutants (Xavier et al., 2013). However, mammalian *Scube2* has recently been shown to be a potent modulator of *lhh* signalling, enhancing *lhh*-stimulated osteoblast differentiation during endochondral bone formation (Lin et al., 2015).

6. Hedgehog reception

6.1. Patched1 and patched2

Ptch1 is first detected in the developing mouse embryo at E7.5 with transcripts present in the ventral neural tube and later, the somites, limb buds and developing craniofacial region (Goodrich et al., 1996; Hahn et al., 1996). In humans, protein-truncating *PTCH1* mutations are responsible for the autosomal dominant Basal Cell Nevus Syndrome (BCNS; also known as the Nevroid Basal Cell Carcinoma Syndrome (NBCCS) or Gorlin–Goltz syndrome) (Wicking et al., 1997), which is characterized primarily by multiple recurrent basal cell carcinomas, recurrent odontogenic keratocysts of the jaws, palmar/plantar pits, ectopic calcification of the falx cerebri and less commonly, CLP and tooth agenesis (Kimonis et al., 1997, 2004, 2013).

Homozygous *Ptch1* mutant mice die between E9.0 and 10.5, with gross phenotypic changes evident at E8. The neural tube fails to close completely and there is overgrowth of the head folds, hindbrain and spinal cord. Embryonic lethality is thought to be secondary to abnormal cardiac development, which has hampered analysis of any potential craniofacial phenotype (Goodrich et al., 1997). However, conditional inactivation of *Ptch1* in CNCC (*Wnt1Cre; Ptch1^{fl/c}*) has established a direct role in the pathogenesis of craniofacial anomalies. Loss of *Ptch1* function in CNCC causes mid-facial expansion and an early defect in the nasal pit, which culminates in cleft lip (Metzis et al., 2013). Interestingly, the wider mid-facial morphology observed in these mutants has been explained by changes in cell packing, specifically a more loosely packed cellular network within the mesenchyme. Cleft lip occurs through a failure of coordinated fusion between medial nasal process, lateral nasal process and maxilla. In *Wnt1Cre; Ptch1^{fl/c}* embryos, these prominences fail to meet; which has been correlated to defective nasal pit invagination, *Ptch1* required in a non-cell-autonomous manner for maintenance of cell shape in the invaginating nasal pit epithelium (Metzis et al., 2013). Unfortunately, even conditional disruption of *Ptch1* in CNCC leads to lethality at E12.0; after primary, but before secondary palate development (Ferguson, 1988; Metzis et al., 2013). It is likely that abnormalities of the secondary palate, such as high-arched and CP would also be observed in these mice, if embryos survived until later in embryogenesis.

Ptch1 is also a key molecule involved in the co-ordination of brain and facial development during mouse embryogenesis. The capability of the C-terminal domain of *Ptch1* to regulate Caspase-9

Table 1
Hedgehog pathway receptor mutations in human craniofacial anomalies.

Gene (Official full name)	Also known as	Location	Exon count	Disease	OMIM	Allelic variants	PMID
BOC cell adhesion associated, oncogene regulated (BOC)	CDON2	3q13.2	23	–	–	–	–
Cell adhesion associated, oncogene regulated (CDON)	CDO CDON1 HPE11 ORCAM	11q24.2	23	Holoprosencephaly-11 (HPE 11)	614226	THR790ALA, 2368A G transition PRO689ALA, 2065C G transversion VAL780GLU, 2339T A transversion SER940ARG, 2818A transversion	21802063 21802063 21802063 21802063
Dispatched homolog 1 (Drosophila)	DISPA	1q41	22	–	–	–	–
Ellis van Creveld (EVC)	EVC1	4p16.2	29	Ellis-van Creveld syndrome	225500	GLN879TER, gln879-to-ter mutation in exon 18 IVS13DS, G-T, +5, substitution in intron 13 ARG340TER, 1018C T transition 1-BP DEL, 734T, 1-bp deletion ARG443GLN, 1328G A transition in exon 10 SER307PRO, 919T C transition in exon 7 1-BP DEL, 2456G, 1-bp deletion	10700184 10700184 10700184 10700184 10700184 10700184
	EVCL DWF-1						
Ellis van Creveld 2 (EVC2)	LBN	4p16.2	24	Weyers acrodermal dysostosis Ellis-van Creveld syndrome	193530 225500	SER307PRO, 919T C transition in exon 7 1-BP DEL, 3660C, frameshift mutation in exon 22 5-BP INS, NT198, 5-bp insertion in exon 1 1-BP INS, 2056C, frameshift in exon 14 ARG399TER, 1195C T transition in exon 10 GLN619TER, 1855C T transition in exon 12 ILE283ARG, missense mutation in exon 7 GLN1009TER, 3754C T transition in exon 18 ARG870TRP, R870W mutation 1-BP DEL, 3793C, 1-bp deletion in exon 22 IVS5, A-G, -2, A-G transition in intron 5 ARG884TER, R884X mutation	10700184 12571802 12571802 12571802 12571802 12468274 12468274 16,404,586 21815252 21815252
	WAD						
				Weyers acrodermal dysostosis	193530	3797T G, LEU1266TER, transversion in exon 22 3797T A, LEU1266TER, transversion in exon 22 GLY1269TER, 3805G T transversion in exon 22 LEU19GLN, c.56T A transversion	19810119 19810119 23220543 25322266
G Protein-Coupled Receptor 161 (GPR161)	RE2	1q24.2	13	Unknown significance [pituitary hormone deficiency (PSIS)?]	262400		
Growth arrest-specific 1 (GAS1)	–	9q21.3-q22	1	Unknown significance [Holoprosencephaly-1 (HPE 1) ?]	236100	GLY259GLU, 775G A transition THR200ARG, 599C G transversion	2058317 2058317
Hedgehog interacting protein (HHIP)	HIP	4q28-q32	13	–	–	–	–
Low density lipoprotein receptor-related protein 2 (LRP2)	DBS GP330	2q31.1	81	Donnai-Barrow Syndrome	222448	TYR2522HIS, 7564T C transition in exon 41 2-BP DEL, 9484GT 2-bp deletion in exon 50 IVS18AS, G-A, -1, G-to-A transition in intron 18 4-BP DEL, 8516TTTA, 4-bp deletion in exon 45 IVS44DS, G-A, +1, G-to-A transition in intron 44 ARG3399TER, 10195C T transition in exon 53 IVS11DS, T-G, +2, T-to-G transversion in exon 11 ARG365TER, 1093C T transition in exon 10 1-BP DEL, NT12437, 1-bp deletion at position 12437 ASP2054ASN, asp205-to-asn mutation	17632512 17632512 17632512 17632512 17632512 17632512 17632512 23033978 23033978
Patched 1 (PTCH1)	PTC BCNS HPE7 PTC1 PTCH	9q22.3	33	Basal Cell Nevus Syndrome	222448	1-BP DEL, 2583C 1-bp deletion within exon 15	8681379

11-BP DEL, NT2442 11-bp deletion within exon 15	8658145
37-BP DEL, NT808 37-bp deletion within exon 6	8681379
2-BP INS, 2047CT 2-bp insertion in exon 13	8681379
1-BP INS, 1-bp insertion in exon 13	8681379
9-BP INS, CODON 815, PRO-ASN-ILE INS	8658145
1-BP INS, 1247T 1-bp insertion in exon 9	12900905
GLN210TER, C-to-T transition in the codon for gln210	8681379
1148G A, G-to-A transition in exon 8	8681379
TRP129TER, 387G A transition in exon 2	22572734
610828 THR728MET, 2171C T transition	11941477
ALA393THR, 1165G A transition	11941477
THR728MET, 2171C T transition	11941477
SER827GLY, 2467A G transition	11941477
THR1052MET, 3143C T transition	11941477
VAL908GLY, 2711G T transversion in exon 17	17001668
605462 PRO-SER, 451C T, transition in exon 3	8782823
605462 IVS20DS, T, +5, somatic splice donor site mutation	9931336
109400 ARG719GLN, heterozygous 2157G A transition in exon 15	18285427
155255 2-BP DEL, NT1170, 2-bp deletion at nucleotide 1170	9931336
-	-
-	-

	NBCCS PTCH11								
					Holoprosencephaly-7 (HPE 7)				
					Basal Cell Carcinoma, Nonsyndromic, included	605462			
					Basal Cell Carcinoma, Nonsyndromic, included	605462			
					Basal Cell Nevus Syndrome	109400			
					Medulloblastoma	155255			
Patched 2 (PTCH2)	PTC2	1p34.1	23						
Scube2 (SCUBE2)	CEGB1 CEGF1 CEGP1	11p15.3	25	-		-	-		-
Smoothened (SMO)	Gx SMOH FZD11	7q32.3	13	-		-	-		-

(Casp9)-associated mitochondrial cell death has a seemingly direct effect on size of the forebrain and the adjacent nasal processes (Aoto and Trainor, 2014). Interestingly, the X-linked inhibitory apoptosis protein (XIAP), which is observed in primary cilia in a Hedgehog-dependent manner, acts as an inhibitor of *Ptch1*-induced cell death. Orchestration of *Ptch1*-induced apoptosis by XIAP in primary cilia protects *Ptch1* from translocation into mitochondria, providing an important survival mechanism in this organelle. This mechanism may also help co-ordinate brain and facial development, with these observations supporting the notion that mitochondrial dysfunction may be a developmental risk factor associated in the pathogenesis of HPE (Aoto and Trainor, 2014).

N-ethyl-N-nitrosourea (ENU)-induced mutagenesis provides an unbiased forward genetic approach for identifying novel alleles important for embryogenesis. In a recent screen for recessive mouse mutations affecting craniofacial morphology, a single nucleotide change was identified at the 3'-end of *Ptch1* exon 13. These mice (*Ptch1^{DL}*, termed DL: Dogface-Like) display abnormal skull and snout morphology and craniosynostosis of the lambdoid suture. Skeletal defects related to the pathology of BCNS were also present, including the scapula, ribcage, secondary palate, cranial base and cranial vault. The general overgrowth, rhabdomyosarcomas and medulloblastomas observed in *Ptch1* heterozygous mice (Goodrich et al., 1997; Hahn et al., 1998) are not seen in *Ptch1^{DL}* mice. This mutation therefore represents a hypomorphic allele of *Ptch1* with the potential to further assess the role of Hedgehog signalling in multiple developmental events, particularly those regulating facial shape (Feng et al., 2013). An additional ENU screen in mice has produced further novel alleles critically required for early craniofacial development, including the *wiggable* mutant, so-called because of the excessive leaf-like laminae or folia in the brain, which resemble a wig (Sandell et al., 2011). Genome sequencing revealed a T to A nucleotide change in intron 15 of *Ptch1* in this mutant, which created a new splice acceptor site, resulting in a premature stop codon in exon 16 and generation of a truncated protein. *Ptch1^{wiggable}* mice die *in utero* at around E12.0 as a result of various defects, including open neural tube and hypertelorism of the face, consistent with a gain-of-function in Hedgehog signalling (Kurosaka et al., 2014; Sandell et al., 2011). In an effort to rescue the phenotype observed in *Ptch1^{wiggable}* mice, compound mutants were generated lacking function of the Hedgehog acyltransferase (Hhat), responsible for modifying Hedgehog proteins through the addition of palmitic acid and essential for appropriate long-range signalling. *Hhat^{creface}* mutants have a phenotype consistent with a loss of Shh signalling, including HPE, acrania and agnathia (Dennis et al., 2012). Interestingly, *Hhat^{creface}; Ptch1^{wiggable}* mice have cleft lip and fissure of the premaxillary bone at E16.5, demonstrating an important role for *Hhat* and *Ptch1* in regulating Shh signalling in the FNP during lip development, specifically through the restriction of canonical Wnt signal activity in the lambdoid region (Kurosaka et al., 2014).

In addition to engineered and induced *Ptch1* mutants, spontaneous mutants have also been reported. A recessive *Ptch1* mouse mutation, mesenchymal dysplasia (*mes*) causes excess skin, increased body weight and mild preaxial polydactyly, with these mice also having a shortened face, wide-set eyes and dome head (Makino et al., 2001). *Ptch1^{D11}* is another spontaneous mutation caused by an aberrant recombination event during production of a *Ptch1* null allele. The *Ptch1^{D11}* locus presumably results in a weak *Ptch1* allele, with homozygous animals being sterile, but otherwise normal (Oro and Higgins, 2003).

In humans, *PTCH1* maps to chromosome 9q22.3 and is widely assumed to be a tumour-suppressor gene (Hahn et al., 1996). Mutation analysis of *PTCH1* in both familial and sporadic HPE cases has revealed four different missense mutations in five unrelated individuals. These findings demonstrate that mutations in

different components of the Shh pathway that lead to a common effect on Shh signalling can each result in the same phenotype: decreased Shh activity causing HPE and increased activity causing tumours (Goodrich et al., 1999; Ming et al., 2002).

Vertebrates have an additional Patched receptor *Ptch2*, which is a structural homologue of *Ptch1* (Motoyama et al., 1998). *Ptch2* mutant mice are viable, fertile and do not display obvious developmental defects, although males develop skin lesions associated with alopecia and ulceration, with progressing age (Nieuwenhuis et al., 2006). However, *Ptch2* is a target of Hedgehog signalling and participates in ligand-dependent feedback inhibition, which in conjunction with *Ptch1* (and *Hhip1*) is an important antagonist of pathway activity, in the neural tube at least (Holtz et al., 2013).

7. Gas1, Cdo and Boc

Gas1, Cdon and Boc are a diverse group of proteins that act as co-receptors within the context of Hedgehog signalling, retaining the ability to interact directly with Shh (Lee et al., 2001a, 2001b; Martinelli and Fan, 2007; McLellan et al., 2008; Okada et al., 2006; Tenzen et al., 2006) and form high-affinity individual complexes with *Ptch1* on the surface of receiving cells (Bae et al., 2011; Izzi et al., 2011). Boc and Cdon are also able to bind Gas1, although these interactions are unlikely to be tripartite (Bae et al., 2011; Izzi et al., 2011); whilst, Cdon and Boc can complex with each other through their extra- and intra-cellular domains (Kang et al., 2002). Collectively, these co-receptors are essential for vertebrate hedgehog signalling; binding of Shh to *Ptch1* alone is not sufficient for pathway activation (Izzi et al., 2011) and mice with collective targeted disruption of *Gas1*, *Cdon* and *Boc* lack all Shh transduction except for some very early rudimentary activity (Allen et al., 2011). A prevailing view is that these individual tissue-specific receptor complexes bind Shh, which leads to de-repression of Smo and activation of the pathway, consistent with findings of both redundancy and specific requirements for these co-receptors in different developmental contexts.

The role of these co-receptors during early craniofacial development has been investigated extensively through the generation of single and compound mouse mutants, which demonstrate a variable and background-dependent severity of phenotype (Fig. 5). Loss of *Cdon* can produce a semilobar-type HPE characterized by cebocephalic face (single nostril, ocular hypotelorism and maxillary hypoplasia) or a less severe microform HPE with philtral dysgenesis and maxillary incisor agenesis (Cole and Krauss, 2003; Hong and Krauss, 2012; Zhang et al., 2006). Loss of *Gas1* results in a more consistent microform HPE associated with maxillary incisor fusion and CP (Seppala et al., 2007), whilst *Boc* mutants lack craniofacial abnormalities but do have misguided commissural axon guidance, cerebellum reduction and reduced ipsilateral retinal ganglion cells (Izzi et al., 2011; Okada et al., 2006; Sanchez-Arrones et al., 2013). As might be expected, *Gas1; Cdon* compound mutants have a more severe semilobar HPE associated with a single external nares, fusion of the nasal processes and absence of maxillary and mandibular skeletal elements (Allen et al., 2007; Seppala et al., 2014). In addition, the generation of *Cdon; Boc* mice on a background associated with only microform HPE in the absence of *Cdon* alone, results in a lobar HPE with much more severe craniofacial anomalies (Zhang et al., 2011). More recently, the craniofacial region of *Gas1; Boc* mutants have been analyzed, demonstrating an allele dosage-dependent phenotype. In particular, *Gas1; Boc* mice have lobar HPE and disruption of the corpus callosum, CLP and maxillary incisor agenesis (Seppala et al., 2014). Significantly, loss-of-function mutations in *GAS1* and *CDON* have both been associated with HPE in humans (Bae et al., 2011; Ribeiro et al., 2010).

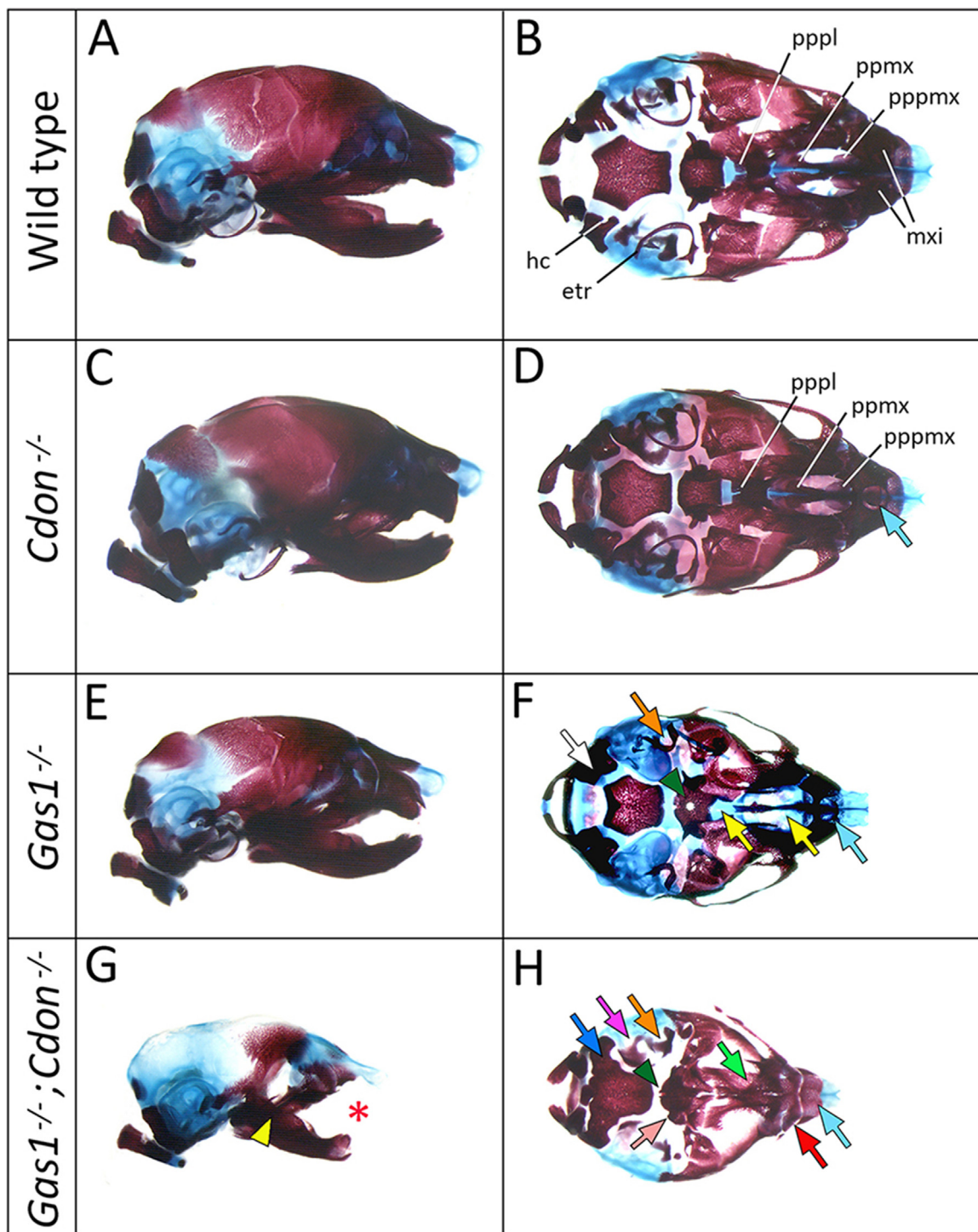


Fig. 5. Craniofacial skeletal defects in mice lacking function of *Cdon* and *Gas1*. Comparison of E17.5 wild type and mutant skulls differentially stained for bone (alizarin red) and cartilage (alcian blue). (A, C, E, G) *Norma lateralis*; (B, D, F, H) *Norma basalis*. The *Cdon*^{-/-} skull is grossly comparable to wild type in size and overall morphology. The palate is intact, but there is only a single maxillary incisor tooth (pale blue arrow). The *Gas1*^{-/-} skull is reduced in size when compared to the wild type and has a number of defects, including single maxillary incisor (pale blue arrow), cleft palate (yellow arrows), fenestration of the neurocranial base (dark green arrowhead), diminutive and poorly formed ectotympanic (orange arrow) and absence of the hypoglossal canals within the exoccipital (white arrow). In the *Gas1*^{-/-}; *Cdon*^{-/-} skull there is a further reduction in size and gross disruption of the skull with maxillary-mandibular stenosis (yellow arrowhead) and a marked open bite occlusion (red *). In addition, the maxillary incisors fail to form (pale blue arrow), there is stenosis within the premaxilla (red arrow), the nasal cavity is ossified (light green arrow), the pterygoid plates are absent (light pink arrow) and the exoccipital-basioccipital bones are fused (dark blue arrow). In addition, the pars canicularis and pars cochlearis are severely disrupted (dark pink arrow) with the ectotympanic fused to the lamina obturans (orange arrow). etm, ectotympanic; hc, hypoglossal canal; mxl, maxillary incisors; ppmx, palatal process of the maxilla; pppmx, palatal process of the premaxilla; pppl, palatal process of the palatine.

An obvious potential influence on the variation that is seen in these loss-of-function co-receptor mouse models is redundancy; however, phenotype may also be influenced by subtle variation in modulating signal activity. *Gas1*, *Cdo* and *Boc* are all negatively

regulated by Hedgehog and as signal levels increase, transcription is progressively reduced (Martinelli and Fan, 2007; Tenzen et al., 2006). A gradient of transcriptional regulation therefore exists within the Shh target field, and as signalling reduces at distance

from the source, transcription of these co-receptors increases, potentially boosting or maintaining signalling in more peripheral tissues. These thresholds of activity may be crucial in ensuring appropriate development, particularly in the craniofacial midline. The situation is further complicated by tissue-specific variation in expression domains between these co-receptors, particularly findings that some can also be expressed in regions of high signal activity (Martinelli and Fan, 2007; Tenzen et al., 2006). Shh can also signal through contact-mediated release in the chick limb, propagated by specialized filopodia that can deliver ligand at a distance (Sanders et al., 2013). Interestingly, Cdon and Boc co-localize in specific micro-domains within these filopodial extensions and whilst these have not been identified in the craniofacial

region, similar mechanisms may also influence signalling here. Finally, these co-receptors have also been shown to function in alternative signalling pathways and therefore potentially play a role in other developmental contexts (Sanchez-Arrones et al., 2012; Segovia and Zarco, 2014).

7.1. Modeling phenotypic variation in HPE through Hedgehog co-receptor function

It has been suggested that in many cases of HPE, clinical variability may be related to a requirement for more than one insult during development and that a combined interaction between multiple gene products and environmental factors determines final phenotypic outcome (Ming and Muenke, 2002; Nanni et al., 1999). Indeed, the analysis of compound Hedgehog co-receptor mutants has demonstrated the importance of genetic interactions on HPE phenotype. Although much less is known about potential environmental factors, fetal exposure to ethanol is a known risk factor for HPE and the mechanistic basis may be through disrupted Shh signalling influencing premature death of CNCC (Ahlgren et al., 2002; Aoto et al., 2008; Li et al., 2007). More recently, these interactions have been modeled using fetal ethanol exposure in mouse embryos engineered with targeted disruption of Hedgehog pathway components, including *Shh* itself, *Ptch1*, *Cdon* and *Gli2*; (Hong and Krauss, 2012, 2013; Kietzman et al., 2014). There is evidence of variation in susceptibility to the effects of ethanol on HPE during murine gestation, depending upon genetic background. These findings further reiterate the complex genetic and environmental interactions that govern outcome in HPE and suggest that Hedgehog receptor function can influence susceptibility to ethanol-induced HPE during development in human populations.

8. LRP2/megalin

Low-density lipoprotein receptor-related proteins (LRPs) are multifunctional cell surface receptors structurally related to the LDL receptor-family. LRP2/megalin encodes an endocytic transmembrane protein found primarily on the apical surfaces of epithelia within the kidney, lung and brain (Kantarci et al., 2007). *Lrp2* has been shown to play a critical role in murine forebrain development (Spoelgen et al., 2005), representing an auxiliary docking site for Shh in the ventral midline of the rostral diencephalon. It locally sequesters Shh, enabling target cell reaction to low concentrations of ligand over a critical time-period during early neurulation (Christ et al., 2012). This role in relation to Shh activity in the forebrain is consistent with craniofacial abnormalities observed when *Lrp2* function is disrupted. Genetic deficiency of this multifunctional receptor in mice results in a HPE phenotype, characterized by abnormal development of the forebrain, absence of olfactory apparatus and abnormalities of facial structures that

originate from forebrain-derived CNCC and contiguous mesoderm (Willnow et al., 1996). In humans, mutations in *LRP2* are seen in subjects presenting with Donnai-Barrow (DBS) syndrome (Kantarci et al., 2007), which have microform HPE characterized by corpus callosum anomalies and enlarged globes, with small colobomas at the optic nerve heads (Kantarci et al., 2007, 1993; Rosenfeld et al., 2010).

9. Gpr161

Gpr161 is a GPCR involved in mediating the ability of cells to sense and respond to their environment (Matteson et al., 2008). The binding of ligands to GPCRs activates heterotrimeric G proteins at the plasma membrane where most of their effectors are intrinsically or transiently associated (Gainetdinov et al., 2004; Garcia-Regalado et al., 2008). Gpr161 acts as a negative regulator of the Hedgehog pathway, fine-tuning PKA-dependent generation of GliR activity through the modulation of cyclic adenosine monophosphate (cAMP) levels (Hwang and Mukhopadhyay, 2014; Mukhopadhyay and Rohatgi, 2014; Mukhopadhyay et al., 2013).

Gpr161 is expressed in several tissues during murine development, initially restricted to the neural folds along the antero-posterior axis. Later, transcripts are detected in the ventricular zone of the developing CNS, forelimb, hindlimb, retina and throughout all stages of lens development (Matteson et al., 2008). Positional cloning has demonstrated that the previously characterized hypomorphic *vacuolated lens* (*vl*) mutation (Wilson and Wyatt, 1986, 1988) is caused by a deletion in *Gpr161* (Matteson et al., 2008). Different neural tube phenotypes are observed in mouse embryos displaying *vl* mutations, with around half displaying frank lumbar-sacral spina bifida; whilst in the remainder, the neural tube closes but dorsal phenotypes are observed (Matteson et al., 2008; Wilson and Wyatt, 1986, 1988). The *vl* mutation leads to lethality in half of homozygous mutants; however, all surviving adult *vl/vl* mice display congenital cataracts and no obvious signs of spina bifida (Matteson et al., 2008).

Recently, a null mutation of *Gpr161* has been shown to produce lethality in mice by E10.5, associated with extensive craniofacial abnormalities and open forebrain/midbrain regions (Mukhopadhyay et al., 2013). In humans, whole-exome sequencing of a family with two siblings displaying pituitary stalk interruption syndrome (PSIS) revealed a unique homozygous missense mutation in *GPR161*. PSIS is a congenital defect of the pituitary gland characterized primarily by a triad of very thin/interrupted pituitary stalk, ectopic (or absent) posterior pituitary gland and hypoplasia/aplasia of the anterior pituitary. Patients with PSIS may present midline defects and various pituitary endocrine deficiencies, ranging from isolated growth hormone deficiency to combined pituitary hormone deficiency (Gutch et al., 2014; Karaca et al., 2014).

10. Smoothened

A single mammalian *Smo* encodes a seven transmembrane GPCR-like protein; however, despite this structure and its role as an activator of the Hedgehog pathway, *Smo* does not bind Shh (Stone et al., 2006) or participate in binding of Shh to *Ptch1* (Chen and Struhl, 1998). *Smo* expression shows considerable overlap with *Ptch1* in the craniofacial region, being adjacent to *Shh*-expressing cells in the developing lens, jaws, tongue, taste buds and teeth (Choi et al., 2014; Stone et al., 2006). Between E11.5–15.5, transcriptional activity is observed in the epithelium and mesenchyme of both maxillary and mandibular processes and strong expression is detected in Meckel's cartilage and the whisker

follicles (Du et al., 2012). *Smo* mutants do not survive beyond E9.5, exhibiting significant ventral cyclopia and HPE, consistent with an essential role in Shh signal transduction. Moreover, these mutants fail to undergo embryonic turning, closure of the ventral midgut and normal heart looping (Zhang et al., 2001).

Smo function is essential in post-migratory CNCC for normal development of the craniofacial skeleton. In zebrafish, loss of *smo* results in a significant disruption of dorso-ventral patterning in the pharyngeal arch skeleton (Eberhart et al., 2006; Swartz et al., 2012). Targeted disruption of *Smo* in murine CNCC disrupts Fox gene expression and appropriate growth and patterning in the early facial primordia; whilst constitutive activation (*SmoM2*) in CNCC produces hyperplasia of the facial processes and gross disorganization, with a failure of most craniofacial skeletogenesis (Jeong et al., 2004). Conditional loss of *Smo* function in the oral epithelium also leads to a reduction in size of the incisors and fusion of the molar dentition in association with morphological disruption and impaired amelogenesis (Gritli-Linde et al., 2002). Further conditional mouse mutants have also helped to elucidate the importance of *Smo* during other aspects of craniofacial development. Inactivation of *Smo* in the otic epithelium leads to cochlear and saccular agenesis as a consequence of impaired Shh signalling in the ventral otocyst; however, no vestibular phenotypes are observed, suggesting that Shh signalling in the dorsal otocyst is dispensable for vestibular morphogenesis (Brown and Epstein, 2011). Moreover, early loss of *Smo* in the lens leads to a distinct microphthalmia (Choi et al., 2014), a phenotype similar to mice lacking function of *Cdo* and *Gas1* (Lee et al., 2001a, 2001b; Zhang et al., 2009).

11. EvC and EvC2

EvC and EvC2 encode transmembrane proteins tethered to the base of the cilium within a complex that positively regulates Hedgehog signalling through a direct interaction with Smo that ultimately promotes formation of GliA (Dorn et al., 2012; Pusapati et al., 2014; Ruiz-Perez et al., 2007). Mutation in either gene causes Ellis van Creveld syndrome, an autosomal-recessive chondroectodermal dysplasia characterized by skeletal and craniofacial anomalies, including severe truncation of the limb and rib skeletons, postaxial polydactyly, multiple intra-oral frenulae and significant disruption to the dentition (Galdzicka et al., 2002; Ruiz-Perez et al., 2000, 2003). The phenotype associated with mutations in either gene are indistinguishable, confirming that they act in a common pathway (Blair et al., 2011); whilst mutations have also been linked to Weyers acrofacial dysostosis (WAD), an autosomal dominant disorder characterized by a similar phenotype to Ellis van Creveld, but with milder expression (D'Asdia et al., 2013; Ruiz-Perez and Goodship, 2009; Ruiz-Perez et al., 2000).

As might be expected, *EvC* is strongly expressed in the developing skeleton and orofacial region during murine embryogenesis and post-natal development. Targeted disruption of *EvC* in the mouse produces an Ellis van Creveld-like chondrodysplasia with diminished Ihh signalling and abnormal growth plate development in the long bones; in particular, premature differentiation of columnar to hypertrophic chondrocytes (Ruiz-Perez et al., 2007). Interestingly, these mice have normal signalling in the neural tube and only slight reduction in the limb bud, suggesting that ciliary *EvC* function is tissue-specific during development (Caparros-Martin et al., 2013). Recently, the ciliary proteins EF-hand calcium-binding domain-containing protein 7 (EFCAB7) and IQ-domain containing protein E (IQCE) have been identified, which anchor *EvC*-*EvC2* to the base of the cilium and therefore promote Hedgehog signalling. In the absence of this tethered complex, Smo can still enrich in the cilium in response to signalling, but fails to

activate Gli2. However, it does retain the ability to regulate Gli3R, which implies some bifurcation of signal transduction downstream of Smo and some independent regulation of GliA and GliR activity (Pusapati et al., 2014).

EvC mutant mice also have dental anomalies, including abnormal patterning, morphogenesis and differentiation, which manifests as dental hypoplasia, molar fusion and defective root development (Nakatomi et al., 2013). *EvC* is expressed in cells responding to Shh signalling in the tooth and the gross dental phenotype is reminiscent of that seen in teeth lacking Shh function in different compartments of the tooth (Cho et al., 2011; Dassule et al., 2000; Gritli-Linde et al., 2002; Jeong et al., 2004). Interestingly, there is a progressive loss of Shh signalling in the developing first molar teeth of the mutant, occurring in a buccal (outer) to lingual (inner) direction and with a corresponding buccal shift of the primary enamel knot, an important signalling centre in the developing tooth. This temporospatial disruption of Shh signalling is the primary source of the dental anomalies that are seen in this mutant. It is interesting that this contrasts with observations in molar tooth germs lacking Shh signalling in dental epithelium, which have reduced signalling and growth retardation in the lingual epithelium (Dassule et al., 2000).

12. Gli processing

Hedgehog signal output is ultimately dictated by the relative balance between positive (GliA) activation and negative (GliR) repression, which has been referred to as the Gli code (Ruiz i Altaba, 1997; Ruiz i Altaba et al., 2007). This balance is further influenced by transcriptional activity; primarily, positive feedback through *Gli1* activation and negative feedback through *Ptch1* (Aberger and Ruiz, 2014). Gli processing also requires a functional primary cilium (Haycraft et al., 2005) and the ciliopathies demonstrate how important this process is for normal development, albeit with significant tissue-specific variation (Badano et al., 2006). However, relatively little is known about how post-translational modification of Gli proteins contributes to the dynamic processes underlying formation of the craniofacial region. Interestingly, analysis of the *talpid²* avian ciliopathic mutant has shown the importance of appropriate Gli processing during facial development. Post-translational processing of both Gli2 and Gli3 are disrupted in the facial prominences of these mutants, with increased levels of Gli3A in the nucleus of these cells resulting in disrupted growth of the facial prominences and clefting (Chang et al., 2014). There is also evidence that the Gli code can be influenced by non-Hedgehog signals, including tumour-suppressors (Stecca and Ruiz i Altaba, 2009) and oncogenes (Stecca et al., 2007). It will be important to understand how other genetic factors and indeed, the environment may influence post-transcriptional activity of Gli proteins, particularly in relation to the craniofacial region.

13. Conclusions

The Shh signalling pathway regulates many aspects of embryonic development, including that of the early craniofacial region. Multiple novel proteins are involved in mediating appropriate signal levels and a number of robust developmental models now exist that provide insight into the role of this pathway during development of this region. Mutations in many of the genes that encode these proteins have now been associated with craniofacial anomalies in human populations.

Many questions relating to Hedgehog signal transduction and developmental disorders remain to be answered. In the

craniofacial region, the relationship between pathway disruption and phenotypic outcome is poorly understood and highly relevant; very obviously in relation to HPE, but also during development of the upper lip and palate. Moreover, little is known about protein distribution and the different tissue-specific requirements of Hedgehog pathway components, particularly the co-receptors. Whilst, our knowledge of post-translational Gli processing and the influence that this process has on normal craniofacial development is very poorly understood.

It is likely that the next decade will continue to see advances in our understanding of these and other questions, particularly as biochemical knowledge of the signalling pathway is extended. The complex gene networks that exist between Hedgehog and other pathways are likely to be further elucidated, as is the role of this pathway in influencing cellular function during craniofacial development. This will ultimately lead to advances in diagnosis, prevention and therapeutic intervention for individuals affected by these craniofacial disorders.

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